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(54) A METHOD FOR CHARACTERIZING SAMPLES BY DETERMINATION OF A FUNCTION OF AT LEAST ONE SPECIFIC PHYSICAL PROPERTY OF PARTICLES OF SAID SAMPLE

EIN VERFAHREN ZUR PROBENCHARAKTERISIERUNG DURCH BESTIMMUNG EINER FUNKTION MINDESTENS EINER SPEZIFISCHEN EIGENSCHAFT DER PROBE

PROCEDE DE CARACTERISATION D'ECHANTILLONS PAR LA DETERMINATION D'UNE FONCTION D'AU MOINS UNE PROPRIETE PHYSIQUE SPECIFIQUE DE PARTICULES DE CES ECHANTILLONS

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Description

[0001] The present invention relates to a method for characterizing samples by determination of a function of at least one specific physical property of units of said sample.

5 [0002] The essence of a number of pharmacological, biological and chemical problems is to detect substances in a sample or to measure the interaction or reaction of these substances. In order to measure the substances in a sample more specifically, usually at least one of the reactants is radioactively or luminescently labelled. A convenient and sensitive type of labels are fluorescent labels.

10 [0003] Widely used methods to monitor interactions by fluorescence are the determination of changes in overall fluorescence intensity or in anisotropy of fluorescence. However, a number of side effects, such as surface binding or fluorescence from impurities, often lead to interpretation problems and artifacts. A second reason which has induced interest towards refined methods of analysis is the need to work with small amounts of a large number of samples in the field of high throughput screening and large capacity diagnostics.

15 [0004] New opportunities for assay development were opened when the technology for monitoring fluorescence from single fluorophore molecules became available. The first successful studies on fluorescence intensity fluctuations were performed by Magde, Elson and Webb (Biopolymers, Vol. 13, 29-61, 1974) who demonstrated the possibility to detect number fluctuations of fluorescent molecules and established a research field called fluorescence correlation spectroscopy (FCS). FCS was primarily developed as a method for determining chemical kinetic constants and diffusion coefficients. The experiment consists essentially in measuring the variation of the number of molecules of specific 20 reactants in time in a defined open volume of solution. Microscopic fluctuations of the concentration of the reactant are detected as fluorescence intensity fluctuations from a small, open measurement volume. The measurement volume is defined by a focussed laser beam, which excites the fluorescence, and a pinhole in the image plane of the microscope collecting fluorescence. Intensity of fluorescence emission fluctuates in proportion with the changes in the number of fluorescent molecules as they diffuse into and out of the measurement volume and as they are created or eliminated 25 by the chemical reactions. Technically, the direct outcome of an FCS experiment is the calculated autocorrelation function of the measured fluorescence intensity.

30 [0005] An important application of FCS is to determine concentrations of fluorescent species having different diffusion rates in a mixture. In order to separate the two terms corresponding to translational diffusion of two kinds of particles in the autocorrelation function of the fluorescence intensity, at least about a two-fold difference in diffusion time is needed, which corresponds generally to an eight-fold difference in the mass of the particles. Furthermore, if one succeeds in separating the two terms in the autocorrelation function of fluorescence intensity, it is yet not sufficient for determining the corresponding concentrations except if one knows the relative brightness of the two different types of particles.

35 [0006] Possible biophysical applications further demand the ability to analyze complex mixtures of different species. For that purpose, Palmer and Thompson studied higher order correlation functions of fluorescence intensity fluctuations and have outlined methods for determining the number densities and relative molecular brightness of fluorescence of different fluorescent species (Biophys. J., Vol. 52, 257-270, August 1987). Their technique may in principle prove useful in detecting and characterizing aggregates of fluorescently labelled biological molecules such as cell surface receptors, but has a major disadvantage of being rather complex, so that data processing of an experiment including the calculation 40 of high-order correlation functions last hours.

45 [0007] A considerably less complicated method than calculation of high order auto-correlation functions for characterizing mixtures of fluorescent species of different specific brightness is a calculation of higher order moments of fluorescence intensity out of experimentally determined distribution of the number of photon counts. This method was presented by Qian and Elson (Biophys. J., Vol. 57, 375-380, February 1990; Proc. Natl. Acad. Sci. USA, Vol. 87, 5479-5483, July 1990). The method of moments, however, is hardly suitable for characterizing complex samples or selecting between competing models of the sample or checking whether the given model is appropriate.

50 [0008] Further improvements were made according to the disclosure of WO-A-98/16814 published 1998-04-23, ie after the priority date of the present application. This publication describes a method for analyzing samples by measuring numbers of photon counts per defined time interval in a repetitive mode from light emitted, scattered and/or reflected by particles in said sample, and determining the distribution of the number of photon counts per said time intervals, characterized in that the distribution of specific brightness of said particles is determined from said distribution of the number of photon counts. The method can also be applied to study fluorescent samples. This special embodiment is the so called fluorescence intensity distribution analysis (FIDA). While FCS distinguishes between different species according to their diffusion time, FIDA distinguishes between them according to their specific brightness.

55 [0009] Kask et al. describe possibilities for the use of fluorescence correlation spectroscopy in the nanosecond time range (Eur. Biophys. J., 12: 163-166, 1985). However, there is no discussion on determining a distribution of a specific physical property from the measured photon interval distribution function in their report.

[0010] Keller et al. (Applied Spectroscopy, vol. 50, no. 7, p. 12A - 32A, 1996) disclose methods for single-molecule

fluorescence analysis in solution.

[0011] Madrazo et al. (Applied Optics, vol. 33, no. 21, p. 4899 - 4905, 1994) disclose a time-interval-statistics method that is based on the measurement of the Laplace transform of the probability function of the time intervals between two successive photoelectrons. This method has been applied to experiments of light diffusion from low-polydispersity samples from which the scattered intensity is weak.

[0012] An object of the invention is to provide a reliable and fast method for characterizing samples.

[0013] The object of the present invention is solved with the method having the features of claim 1.

[0014] In the following description reference is made to the drawings wherein:

Figures 1 and 2 show graphs related to the below described Examples 2.

[0015] It is to be understood that the following description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the following description. By way of example, the invention will be described primarily with reference to monitoring numbers of photon counts from light emitted by fluorescently labelled particles in a sample. This is because fluorescence is a very sensitive means allowing to monitor single molecules, and still rather selective allowing to distinguish between different species.

[0016] The term "unit of a sample" refers, in general, to subparts of the sample which are capable of emitting, scattering and/or reflecting radiation. A sample might contain a number of identical units or different units which preferably can be grouped into species. The term "different species" refers also to different states, in particular different conformational states, of a unit such as a molecule. Fluorescently labelled or naturally fluorescent molecules, molecular complexes, vesicles, cells, beads and other particles in water or other liquids are examples of fluorescent units in liquid samples, while examples of fluorescent units or particles of a solid sample are impurity molecules, atoms or ions, or other fluorescence centers.

[0017] What is meant by the term "specific physical property" is generally a physical measurable property having a certain value or interval of values for one species and, in general, another value or interval of values for another species if said species have been characterized on basis of said specific physical property. Examples of specific physical properties are: diffusion coefficient, absorption cross-section, quantum yield of fluorescence, specific brightness, anisotropy of fluorescence, fluorescence decay time, ratio of fluorescence intensity passing through different optical filters, etc.

[0018] The specific brightness in the sense of the present invention is a physical characteristic which expresses in what extent a unit of given species is able to emit, scatter and/or reflect radiation. It is thought to characterize single units and therefore the value of specific brightness is neither depending on concentration of the units, nor on the presence of other units. Thus, a change of the total count rate of photons emitted, scattered and/or reflected from the measurement volume, if only due to a change in concentration of the total number of units, does not influence the value of specific brightness. Specific brightness of a unit is usually expressed in terms of the mean count rate per unit which is a weighted average of the count rate over coordinates of the unit in the measurement volume. In some cases, one might prefer to express the specific brightness in count rates corresponding to a unit positioned in a place where the count rate has its top values. This could e.g. be the center of the focus of an incident beam.

[0019] According to the invention, a very rapid and reliable characterization of samples having units which emit, scatter and/or reflect radiation is possible. In a first step, the length of time intervals, between photon counts is measured in a repetitive mode. In a second step, a function or a series of functions of the length of said time intervals is determined. In a third step, a function of at least one specific physical property of said units is determined on basis of said function or said series of functions of the length of time intervals. The latter determination is performed by finding a close fit between the experimentally determined and a theoretical function or series of functions of the length of said time intervals; the latter of which takes into account parameters of the spatial brightness function characteristic for the optical set-up which is to be used within experiments.

[0020] The spatial brightness function might e.g. be accounted for in analysis in the following way. Theoretical expressions of the photon interval distribution (corresponding either to consecutive photon counts or to photon counts with a certain number of intermediate photon counts) contain integrals over spatial coordinates of functions of spatial brightness function. For example, the cumulative distribution function of time intervals between consecutive counts looks as follows:

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$$P(\tau) = 1 - \frac{1}{\Omega} \exp \left\{ \sum_i C_i \int_V [\exp(-Q_i B(x)\tau) - 1] dV \right\} \sum_i C_i Q_i \int_V B(x) \exp(-Q_i B(x)\tau) dV,$$

where

$$\Omega = \sum_i C_i Q_i \int_v B(x) dV.$$

5

with

- P(τ): cumulative distribution function of τ
 10 τ : time interval between two consecutive photon counts
 C_i: concentration of i-th species
 V: volume
 Q_i: specific brightness of i-th species
 B(x): brightness as a function of spatial coordinates

15

[0021] In real situations, the spatial brightness function B(x) often has a rather complicated form and its determination would be extremely inconvenient. According to the invention, this difficulty has been overcome by replacing the integration over spatial coordinates by integration over volume elements of given brightness. Even though the volume-brightness distribution is initially unknown for a given equipment, it has turned out to be possible to determine it empirically, with a sufficient accuracy for time interval distribution analysis (see example below).

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[0022] What characteristics of the spatial brightness function can be employed when determining the expected/theoretical function or series of functions of the length of time intervals are values of volumes of the sections of the measurement volume corresponding to a selected set of values of the spatial brightness. Typically, a set of twenty or thirty values of the spatial brightness positioned at a constant distance from each other in the logarithmic scale have been selected, covering two or three orders of magnitude. Contributions from the lower brightness areas can be accounted for by a single parameter, their relative contribution to fluorescence intensity. Intensity fluctuations of this light can be neglected. Because of the large number of the sections of the measurement volume, it would be less preferred to consider volumes corresponding to each of the sections as independent variables. It is convenient to consider them as variables depending on a few other parameters, and determine the values of these parameters which yield a close fit between the experimentally determined and the calculated distribution of the length of time intervals. Conveniently, a relatively simple model of the optical set-up is applied, which is not accounting for aberrations of the optics used, and which determines volumes of the sections of the measurement volume. For instance, the volumes of the sections depend on values of the convergence angle of the laser beam and the diameter of the pinhole. It might therefore be preferred to use the pinhole dimensions and the convergence angle of the incident laser beam as modelling parameters of the spatial brightness function.

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[0023] Alternatively, simple mathematical expressions with formal parameters can be used instead of physical models for determining the volumes of spatial sections. The values of the formal parameters should preferably be selected in such a way that a close fit between experimental and theoretical functions of the length of time intervals is achieved. Formal flexible expressions are advantageous because they yield a good fit between experimental and theoretical functions of the length of time intervals. Secondly, calculations based on simple mathematical expressions are very fast compared to those based on physical models.

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[0024] According to the invention it is preferred that the length of time intervals between consecutive photon counts are measured in a repetitive mode.

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[0025] It might also be preferred to measure the length of time intervals between photon counts separated by a given number of intermediate photon counts. A series of functions might be built by measuring time intervals between photon counts separated by different numbers of intermediate photon counts. In such a series, one might e.g. measure the length of time intervals between consecutive photon counts, between photon counts separated by one intermediate photon count, between photon counts separated by two intermediate photon counts, etc.

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[0026] In a further preferred embodiment, said function of at least one specific physical property of said units and/or said function of the length of said time intervals is a distribution function.

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[0027] It might be preferred to monitor the fluctuating intensities of radiation emitted, scattered and/or reflected by said units in at least one measurement volume with the help of only one detection means. If one is interested in characterization of species according to more than one specific physical property, for example, to different polarizations or spectral sensitivities of fluorescence detection, then it might be preferred to use more than one detection means. In a further embodiment, a plurality of assays might be performed simultaneously, requiring a parallelization of detection means. Any detector which is capable to detect radiation emitted, scattered and/or reflected by units of the sample may be used. Appropriate detection means such as an avalanche photodiode, a photomultiplier or conventional photodiodes are well known to those of skill in the art. It can also be preferred to use a multidetector consisting of a

monolithic configuration of a plurality of detectors, especially if one wants to measure a set of samples in parallel as it is the case in miniaturized high throughput screening. It can further be preferred to use a two-dimensional multiarray detector.

[0028] According to a preferred embodiment, the units or particles are molecules, aggregates, vesicles, cells, viruses, bacteria, centers, or mixtures thereof in solids, liquids or gases. It may be preferred to group units into species which can be distinguished by at least one of their specific physical properties. At least one of the species can be luminescent, preferably fluorescent, and/or can be luminescently labelled.

[0029] According to the invention, the specific physical property characterizing said units is the specific brightness.

[0030] It may further be preferred to characterize fluorescent units by the polarization ratio of their fluorescence, or fluorescence anisotropy, or any other property expressing the extent of polarization of fluorescence.

[0031] In one embodiment, a further specific physical property characterizing the fluorescent units is the ratio of fluorescence intensities corresponding to different excitation wavelengths and/or different spectral sensitivities of fluorescence detection, or any other property expressing the dependence of fluorescence intensity on the wavelength of excitation and/or detection.

[0032] In a further preferred embodiment, a further specific physical property characterizing said fluorescent units is lifetime of fluorescence.

[0033] It can further be preferred to characterize said units by their diffusion coefficient, or correlation time of radiation intensity fluctuations, or any other property directly related to said diffusion coefficient.

[0034] The specific physical properties, in particular luminescence properties like fluorescence lifetime or fluorescence anisotropy, of the units can be varied by conjugating them with a specific luminophore via different linker molecules. It may be preferred to use polymeric linker molecules consisting of a varying number of equal or different monomers.

[0035] The luminescence properties of the units may also be varied by conjugating them with a first molecule, as e.g. biotin, which binds a luminescently labelled second molecule, as e.g. luminescently labelled avidin or streptavidin.

[0036] The luminescence properties of a particle can also be changed by energy transfer. Energy absorbed by a donor is transferred upon close contact to a luminophor of an acceptor and subsequently emitted.

[0037] In a preferred embodiment, the measurement volume is only a part of the total volume of the sample and said units are diffusing and/or being actively transported into and out of said measurement volume and/or said sample is actively transported and/or optically scanned. If said units, e.g. fluorescent particles, are sufficiently small, then diffusion is fast enough for data acquisition from a great number of counting intervals. However, if the characteristic time of diffusion is substantially longer than the time interval for measuring fluorescence intensity, then active transport (flow or scanning) can considerably save time of data acquisition.

[0038] The method according to the present invention is particularly well suited for high throughput screening, diagnostic purposes, monitoring polymerization, aggregation or degradation processes, or for general analytical purposes, such as environmental analytics or process control.

[0039] In screening procedures, substances that are possibly pharmacologically active can be analyzed through their interaction with specific receptors by examining said interaction with binding of a luminescently labelled ligand to receptors wherein natural receptors on their carrier cells as well as receptors on receptor-overexpressing carrier cells or receptors on vesicles or receptors in the form of expressed molecules or molecular complexes may be used. Moreover, the interaction of substances with enzymes in solution or in their genuine cellular environment can be detected by monitoring a change of the substrate of the enzyme, e.g. a change in size, brightness, rotational diffusion, or any other of the above mentioned fluorescence properties. Another means of determining enzyme activity is to add a fluorescently labelled molecule, which binds to either educt or product of the enzymatic reaction. Another method for investigating pharmacological activity of substances is the measurement of reporter systems such as Green Fluorescent Protein (GFP) expression, and of the properties of molecules to which GFP is attached. Further applications, especially concerning the performance of assays, are disclosed in WO-A-94/16313.

[0040] For the detection of specific recognition reactions, potential active substances can be present in complex natural, synthetic or semisynthetic mixtures which are subjected to separation prior to analysis. These mixtures can be separated first e.g. by chromatography to test the individual fractions for the presence of functional compounds preferably "on line" in a capillary at the end of a separation matrix. The coupling of fractionating methods with FCS detection is described in detail in WO-A-94/16313.

[0041] Often, aggregation and degradation are phenomena to be monitored. Aggregates display e.g. brightnesses and diffusion times different from the monomers. In determining both properties, the measurements become more precise.

[0042] In sequencing according to the method of Sanger, oligomers of different length, of which the terminating nucleic acid is labelled with a dye, are identified. Advanced techniques, as e.g. the one described in DE-A-38 07 975, use dyes which exhibit different properties, such as fluorescence lifetime, according to the type of base they are attached to. The determination of a base is much more safe if several properties, such as fluorescence lifetime and

brightness, or any other specific physical property, are determined according to the invention and cross checked for consistency. In a preferred embodiment, the sample to be sequenced is separated by gel or capillary electrophoresis, or a separation step is conducted by capillary electrochromatography, electrohydrodynamic migration or related electrokinetic methods.

- 5 [0043] In one embodiment, said function of the length of time intervals is fitted using a priori information on said sample. In a further embodiment, said function of the length of time intervals is processed by applying an inverse transformation with linear regularization (ITR) or inverse transformation with constraints (ITC) or inverse transformation with regularization and constraints (ITRC). Inverse transformation can be used to determine which composition of the sample would yield the theoretical values of the function of the length of time intervals close to the experimental data.
- 10 Because of statistical errors and limited sizes of measured data, inverse transformation is often an ill-posed mathematical problem, characterized by wild oscillations in its outcome. ITR, ITC and ITRC stabilize the mathematical problem by looking for a "regular" (e.g. a smooth) or constrained solution, for example by minimizing the sum of squared deviations of statistical data and a function of the solution itself, penalizing "irregular", usually irreproducible structures in the outcome, or values having no physical meaning. An example of constraining is disallowing negative values for concentrations (For the method of ITR, see, e.g., W. H. Press, S. A. Teukolsky, W. T. Vetterling, B. P. Flannery, Numerical recipes in C: the art of scientific computing, second edition, Cambridge University Press, 1992, p. 808).
- 15 [0044] In fluorescence studies, it may be advantageous to take measures for reducing the background count rate, arising from Raman scattering in the solute material and dark count rate of the detector, with respect to the count rate per unit. In particular, it is in some cases preferred to use measurement volumes smaller than 10^{-12} l, more preferably smaller than 10^{-14} l.
- 20 [0045] The measurement volumes or samples can preferably be arranged on two-dimensional carriers, such as membranes or sheets having wells. Suitable carrier systems are e.g. described in WO-A-94/16313. In a further preferred embodiment, said measurement volumes can be arranged in a linear way, as e.g. in a capillary
- 25 [0046] Advantageously, the high signal to background count rate and the small optical measurement volume may be achieved by using at least one microscope objective, preferably with a numerical aperture ≥ 0.9 , in a confocal manner for both focussing the incident laser beam and collecting radiation emitted, scattered and/or reflected by units in said sample.
- [0047] A suitable device is disclosed in WO-A-94/16313.
- 30 [0048] In a further preferred embodiment, the measurement volume is restricted by the use of elements of near field optical microscopy. Near field optical microscopy means here that the light passes through an aperture with at least one of its dimensions being smaller than the wavelength of the light used and which is in direct contact to the measurement volume. The aperture may consist of an opaque layer with at least one hole of said diameter or at least one slit of appropriate width and/or a tapered glass fiber or wave guide with a tip diameter of said width, optionally coated with an opaque layer outside. Near field optical microscopy can be used for focussing the excitation light of the units, and/or collecting the light emitted by the units. A suitable device is disclosed in WO-A-96/13744.
- 35 [0049] Another preferred embodiment combines near field optical microscopy for the excitation light path, and conventional optical microscopy for the emission light path, or vice versa. The present invention profits from such a realization in the sense that the size of the measurement volume is reduced compared to conventional confocal microscopy. Thus, the present invention can be used to measure higher concentration of particles as with other optical schemes.
- 40 [0050] In a preferred embodiment of the method, multiple photon excitation is used to excite a unit. Multiple photon excitation means that the sum, difference or any other combination of wave frequencies of two, three or more photons is used for excitation of e.g. luminescence. Such an excitation scheme has an advantage in a sense that the excitation probability is not linearly dependent on excitation intensity, but on the second or higher power. Thus, the multiple photon excitation is mostly limited to the volume of the laser focus, whereas outside the laser focus no spurious excitation is generated. The present invention profits from such an excitation scheme in a sense that less background is generated compared to single photon excitation, and that no pinhole is necessary to restrict the measurement volume. Appropriate laser sources of picosecond or subpicosecond pulses are well known to those of skill in the art.

EXAMPLE 1

- 50 [0051] The way how the distribution of specific brightnesses of fluorescent molecules is determined from experimental time interval distribution is illustrated by this example. Data to be analyzed in this example are computer simulated. This computing method saves much time because it does not require the preparation of many different experimental situations. However, brightnesses of molecules have been selected to correspond to fluorescent molecules in aqueous solutions. When data were simulated, as well as in fitting the data, the uneven spatial brightness distribution characteristic to real equipments was accounted with the help of $N = 30$ volume elements corresponding to 30 different values of spatial brightness. The following formula were used for calculating the spatial brightnesses (B_i) and sizes (V_i) of the volume elements:

$$B_i = \frac{N - i}{N}$$

5 $V_i = a_0(i+1) + a_1(i+1)^2 + a_2(i+1)^3 + a_3\delta(i+1, N)$

$i = 0, \dots, N - 1$

- 10 [0052] In simulations it is not necessary to use realistic values of parameters of spatial brightness function. Nevertheless, numeric multipliers on the right side of the last expression in simulations it is not necessary to use realistic values of parameters of spatial brightness function. Nevertheless, numeric multipliers on the right side of the last expression are selected at values of the corresponding parameters of the spatial brightness function independently determined for the laser-excited fluorescence microscope (ConfoCor®, Carl Zeiss Jena GmbH, Jena & EVOTEC Bio-
15 Systems AG, Hamburg) to be used in experiments (in arbitrary units):

$a_0 = 4.6 \times 10^{-3}$

20 $a_1 = 7.4 \times 10^{-4}$

25 $a_2 = 5.1 \times 10^{-5}$

$a_3 = 1.4$

- 30 [0053] A time interval distribution was simulated for a solution of single fluorescent species at $C = 0.75$ particles per measurement volume and $Q = 150$ kHz per particle. The data were simulated with a time resolution of $0.4 \mu s$, and 100 data points were involved, covering the range of 0 to $40 \mu s$. In curve fitting, at least one of the volume parameters or concentration was fixed because of the arbitrariness of the volume unit. We fixed a_0 . From fitting, the following values of parameters were determined:

- 35 $C = 0.734$ particles per measurement volume
 $Q = 145.2$ kHz per particle

40 $a_1 = 7.35 \times 10^{-4}$

$a_2 = 5.21 \times 10^{-5}$

45 $a_3 = 1.8 \times 10^{-4}$

EXAMPLE 2

- 50 [0054] Another simulated time interval distribution function corresponding to a solution of two fluorescent species, with specific brightnesses of 1000 kHz/particle and 5 kHz/particle, and concentrations of 0.01 particles per measurement volume and 7.0 particles per measurement volume, is presented on Fig. 1, together with its fit curve. Fig. 2 shows the residuals. The simulated curve corresponds to an experiment of 10 s duration. In curve fitting, the values of spatial parameters were fixed. Curve fitting yielded the following values of parameters:

- 55 $C_1 = 0.00978 (\pm 2.8\%)$ particles per measurement volume
 $Q_1 = 1011.8 (\pm 1.5\%)$ kHz per particle
 $C_2 = 7.011 (\pm 19\%)$ particles per measurement volume
 $Q_2 = 5.009 (\pm 19\%)$ kHz per particle

Claims

1. A method for characterizing samples having fluorescent particles, by monitoring fluctuating intensities of radiation emitted by said particles in at least one measurement volume, the monitoring being performed by at least one detection means, said method comprising the steps of:
 - a) measuring in a repetitive mode a length of time intervals between photon counts,
 - b) determining a function or a series of functions of the length of said time intervals,
 - c) determining a function of at least one specific physical property of said particles on the basis of said function or said series of functions of the length of time intervals, wherein a specific physical property is specific brightness, by finding a close fit between the experimentally determined and a theoretical function or series of functions of the length of said time intervals, the latter of which takes into account parameters of the spatial brightness function characteristic for the optical set-up.
- 15 2. The method according to claim 1, wherein said length of time intervals is measured between consecutive photon counts.
3. The method according to claim 1, wherein said length of time intervals is measured between two photon counts separated by a given number of intermediate photon counts.
- 20 4. The method according to at least one of the claims 1 to 3, wherein said series of functions is built by measuring time intervals between two photon counts separated by different numbers of intermediate photon counts,
5. The method according to at least one of the claims 1 to 4, wherein said function of at least one specific physical property of said particles and/or said function of the length of said time intervals is a distribution function.
- 25 6. The method according to at least one of the claims 1 to 5, wherein said particles are molecules, aggregates, vesicles, cells, viruses, bacteria, beads, or mixtures thereof in liquids or gases.
- 30 7. The method according to at least one of the claims 1 to 6, wherein said particles can be grouped into species which can be distinguished by at least one of their specific physical properties.
8. The method according to at least one of the claims 1 to 7, wherein a further specific physical property characterizing said particles is the polarization ratio of their fluorescence, or fluorescence anisotropy, or any other property expressing the extent of polarization of fluorescence.
- 35 9. The method according to at least one of the claims 1 to 8, wherein a further the specific physical property characterizing said particles is the ratio of fluorescence intensities corresponding to different excitation wavelengths and/or different spectral sensitivities of fluorescence detection, or any other property expressing the dependence of fluorescence intensity on the wavelength of excitation and/or detection.
10. The method according to at least one of the claims 1 to 9, wherein a further specific physical property characterizing said particles is lifetime of fluorescence.
- 40 11. The method according to at least one of the claims 1 to 10, wherein a further specific physical property characterizing said particles is the diffusion coefficient, or correlation time of radiation intensity fluctuations, or any other property directly related to said diffusion coefficient.
12. The method according to at least one of the claims 1 to 11, wherein the fluorescence properties of particles are varied by conjugating them with a first molecule, in particular biotin, which binds a fluorescently labelled second molecule, in particular fluorescently labelled avidin or streptavidin.
- 50 13. The method according to at least one of the claims 1 to 12, wherein the fluorescence properties of a particle are changed by energy transfer, in which energy absorbed by said particle is transferred upon close contact to a fluorophore of an acceptor and subsequently emitted.
14. The method according to at least one of the claims 1 to 13, wherein said particles each carry a number of binding sites for fluorescent particles.

15. The method according to at least one of the claims 1 to 14, wherein the measurement volume is only a part of the total volume of said sample and has a volume $\leq 10^{-12}$ l, preferably $\leq 10^{-14}$ l.
- 5 16. The method according to at least one of the claims 1 to 15, wherein said particles are diffusing and/or being actively transported into and out of said measurement volume and/or said sample is actively transported and/or optically scanned.
- 10 17. The method according to at least one of the claims 1 to 16, wherein the measurement volumes or samples are arranged on a two-dimensional carrier, in particular on a membrane or in sheets having wells, or in linear way, preferably in a capillary system.
- 15 18. The method according to at least one of the claims 1 to 17, wherein a confocal microscope set-up is used, comprising at least one microscope objective, preferably with a numerical aperture ≥ 0.9 , for both focussing an incident laser beam and collecting radiation emitted by said particles of said sample, a dichroic mirror, a pinhole in the image plane of said microscope objective, a detection means, a data acquisition means, and optionally means for scanning and/or actively transporting said sample.
- 20 19. The method according to at least one of the claims 1 to 18, wherein said measurement volume is restricted by the use of elements of near field optical microscopy, or their combination with conventional optical microscopy.
- 20 20. The method according to at least one of the claims 1 to 19, wherein fluorescence is induced using multiple photon excitation.
- 25 21. The method according to at least one of the claims 1 to 20, wherein said distribution of the length of time intervals is fitted using a priori information on said sample.
22. The method according to at least one of the claims 1 to 20, wherein said distribution of the length of time intervals is processed by applying an inverse transformation with linear regularization and/or constraints.
- 30 23. The method according to at least one of the claims 1 to 22 for use in high throughput screening, diagnostics, monitoring polymerization, aggregation and degradation processes, particle sorting, or nucleic acid sequencing.

35 **Patentansprüche**

1. Verfahren zur Charakterisierung von Proben, die fluoreszierende Teilchen aufweisen, durch Messung schwankender Intensitäten von Strahlung, die durch diese Teilchen emittiert wird, in wenigstens einem Messvolumen, wobei die Messung durch wenigstens eine Nachweisanordnung durchgeführt wird, wobei das Verfahren die folgenden Schritte umfasst:
- 40 a) das Messen einer Länge von Zeitintervallen zwischen Photonzählimpulsen im Wiederholungsmodus,
b) die Bestimmung einer Funktion oder einer Reihe von Funktionen der Länge dieser Zeitintervalle,
c) die Bestimmung einer Funktion wenigstens einer spezifischen physikalischen Eigenschaft der Teilchen auf der Basis dieser Funktion oder dieser Reihe von Funktionen der Länge von Zeitintervallen, worin eine spezifische physikalische Eigenschaft die spezifische Helligkeit ist, indem man eine enge Übereinstimmung zwischen der experimentell bestimmten und einer theoretischen Funktion oder Reihe von Funktionen der Länge der Zeitintervalle findet, wobei man bei den zuletzt genannten, Parameter der räumlichen Helligkeitsfunktionscharakteristik für den optischen Versuchsaufbau berücksichtigt.
- 45 2. Verfahren gemäß Anspruch 1, worin die Länge von Zeitintervallen zwischen zwei aufeinanderfolgenden Photonzählimpulsen gemessen wird.
3. Verfahren gemäß Anspruch 1, worin die Länge von Zeitintervallen zwischen zwei Photon-Zählimpulsen gemessen wird, die durch eine vorgegebene Anzahl von intermediären Photon-Zählimpulsen getrennt sind.
- 50 4. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 3, worin die Reihe von Funktionen durch Messung von Zeitintervallen zwischen zwei Photon-Zählimpulsen aufgebaut wird, die durch eine unterschiedliche Anzahl von intermediären Photon-Zählimpulsen getrennt sind.

5. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 4, worin die Funktion wenigstens einer spezifischen physikalischen Eigenschaft dieser Teilchen und/oder die Funktion der Länge von Zeitintervallen eine Verteilungsfunktion ist.
- 5 6. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 5, worin die Teilchen Moleküle, Aggregate, Vesikel, Zellen, Viren, Bakterien, Kugelchen oder Mischungen derselben in Flüssigkeiten oder Gasen sind.
- 10 7. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 6, worin die Teilchen in Spezies eingruppiert werden können, die durch wenigstens eine ihrer spezifischen physikalischen Eigenschaften unterschieden werden können.
- 15 8. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 7, worin eine weitere spezifische physikalische Eigenschaft, welche die Teilchen charakterisiert, das Polarisierungsverhältnis ihrer Fluoreszenz oder die Fluoreszenzanzisotropie oder irgendeine andere Eigenschaft ist, die das Ausmaß der Polarisierung der Fluoreszenz ausdrückt.
- 20 9. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 8, worin eine weitere spezifische physikalische Eigenschaft, welche die Teilchen charakterisiert, das Verhältnis von Fluoreszenzintensitäten ist, entsprechend den unterschiedlichen Anregungswellenlängen und/oder unterschiedlichen spektralen Empfindlichkeiten des Fluoreszenznachweises, oder irgendeine andere Eigenschaft, die die Abhängigkeit der Fluoreszenzintensität von der Wellenlänge der Anregung und/oder des Nachweises ausdrückt.
- 25 10. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 9, worin eine weitere spezifische physikalische Eigenschaft, welche die Teilchen charakterisiert, die Lebenszeit der Fluoreszenz ist.
11. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 10, worin eine weitere spezifische physikalische Eigenschaft, welche die Teilchen charakterisiert, der Diffusionskoeffizient oder die Korrelationszeit von Strahlungsintensitätsschwankungen oder irgendeine andere Eigenschaft ist, die mit dem Diffusionskoeffizienten direkt in Beziehung steht.
- 30 12. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 11, worin die Fluoreszenzeigenschaften von Teilchen variiert werden, indem man sie mit einem ersten Molekül paart, insbesondere Biotin, das ein fluoreszenzmarkiertes zweites Molekül bindet, insbesondere fluoreszenzmarkiertes Avidin oder Streptavidin.
- 35 13. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 12, worin die Fluoreszenzeigenschaften eines Teilchens durch Energieübertragung verändert werden, worin durch dieses Teilchen absorbierte Energie in engem Kontakt auf ein Fluorophor eines Akzeptors übertragen wird und anschließend emittiert wird.
- 40 14. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 13, worin die Teilchen jeweils eine Anzahl von Bindungsstellen für fluoreszierende Teilchen tragen.
15. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 14, worin das Messvolumen nur ein Teil des Gesamtvolumens der Probe ist und ein Volumen von $\leq 10^{-12}$ l, vorzugsweise $\leq 10^{-14}$ l hat.
- 45 16. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 15, worin die Teilchen in das Messvolumen diffundieren oder aktiv transportiert werden und aus demselben heraus diffundieren oder aktiv transportiert werden, und/oder die Probe aktiv transportiert wird und/oder optisch abgetastet wird.
17. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 16, worin die Messvolumina oder Proben auf einem zweidimensionalen Träger, insbesondere auf einer Membran oder in Platten mit Nähfischen, oder auf lineare Weise, vorzugsweise in einem Kapillarsystem, angeordnet werden.
- 50 18. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 17, worin eine konfokale Mikroskop-Anordnung verwendet wird, umfassend wenigstens ein Mikroskopobjektiv, vorzugsweise mit einer numerischen Apertur $\geq 0,9$, um sowohl einen einfallenden Laserstrahl zu fokussieren als auch Strahlung zu sammeln, die durch die Teilchen der Probe emittiert wird, einen dichroitischen Spiegel, eine Lochblende in der Bildebene des Mikroskopobjektivs, eine Nachweis-Anordnung, eine Datenaquisitions-Anordnung und gegebenenfalls Anordnungen zum Abtasten und/oder aktiven Transportieren der Probe.

19. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 18, worin das Messvolumen durch die Verwendung von Elementen der optischen Nahfeld-Mikroskopie oder deren Kombination mit konventioneller optischer Mikroskopie eingeschränkt wird.
- 5 20. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 19, worin die Fluoreszenz unter Verwendung mehrfacher Photonenanregung induziert wird.
- 10 21. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 20, worin die Verteilung der Länge von Zeitintervallen unter Verwendung einer *a priori* Information über diese Probe angepasst wird.
- 15 22. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 20, worin die Verteilung der Länge von Zeitintervallen durch Anwendung einer Umkehrtransformation mit linearer Regelung und/oder Beschränkungen verarbeitet wird.
23. Verfahren gemäß wenigstens einem der Ansprüche 1 bis 22 zur Verwendung beim Screening mit hohem Durchsatz, in der Diagnostik, der Polymerisationsüberwachung, bei Aggregations- und Abbauprozessen, der Teilchensortierung oder der Nucleinsäuresequenzierung.

Revendications

- 20 1. Un procédé de caractérisation d'échantillons ayant des particules fluorescentes, par contrôle des intensités fluctuantes de rayonnement émis par lesdites particules dans au moins un volume de mesure, le contrôle étant effectué par au moins un moyen de détection, ledit procédé comprenant les étapes suivantes :
- 25 a) mesurer, dans un mode répétitif, une longueur d'intervalles de temps entre des impulsions photoniques,
b) déterminer une fonction ou une série de fonctions de la longueur desdits intervalles de temps,
c) déterminer une fonction d'au moins une propriété physique spécifique desdites particules sur la base de ladite fonction ou de ladite série de fonctions de la longueur d'intervalles de temps, une propriété physique spécifique étant la luminosité spécifique, en trouvant un ajustement étroit entre la fonction ou série de fonctions déterminée expérimentalement et une fonction ou série de fonctions théorique de la longueur desdits intervalles de temps, cette dernière prenant en compte les paramètres de la fonction de luminosité spatiale caractéristique du montage optique.
- 30 2. Le procédé selon la revendication 1, dans lequel ladite longueur d'intervalles de temps est mesurée entre deux impulsions photoniques consécutives.
- 35 3. Le procédé selon la revendication 1, dans lequel ladite longueur d'intervalles de temps est mesurée entre deux impulsions photoniques séparées par un nombre donné d'impulsions photoniques intermédiaires.
- 40 4. Le procédé selon au moins l'une des revendications 1 à 3, dans lequel ladite série de fonctions est construite en mesurant les intervalles de temps entre deux impulsions photoniques séparées par des nombres différents d'impulsions photoniques intermédiaires.
- 45 5. Le procédé selon au moins l'une des revendications 1 à 4, dans lequel ladite fonction d'au moins une propriété physique spécifique desdites particules et/ou ladite fonction de la longueur desdits intervalles de temps est une fonction de distribution.
- 50 6. Le procédé selon au moins l'une des revendications 1 à 5, dans lequel lesdites particules sont des molécules, agrégats, vésicules, cellules, virus, bactéries, perles ou leurs mélanges dans des liquides ou des gaz.
7. Le procédé selon au moins l'une des revendications 1 à 6, dans lequel lesdites particules peuvent être groupées en espèces qui peuvent être distinguées par au moins une de leurs propriétés physiques spécifiques.
- 55 8. Le procédé selon au moins l'une des revendications 1 à 7, dans lequel une propriété physique supplémentaire caractérisant lesdites particules est le rapport de polarisation de leur fluorescence, ou l'anisotropie de fluorescence, ou toute autre propriété exprimant le degré de polarisation de la fluorescence.
9. Le procédé selon au moins l'une des revendications 1 à 8, dans lequel une propriété physique spécifique supplémentaire caractérisant lesdites particules est le rapport de polarisation de leur fluorescence, ou l'anisotropie de fluorescence, ou toute autre propriété exprimant le degré de polarisation de la fluorescence.

mentaire caractérisant lesdites particules est le rapport des intensités de fluorescence correspondant à différentes longueurs d'onde d'excitation et/ou différentes sensibilités spectrales de détection de fluorescence, ou toute autre propriété exprimant la dépendance de l'intensité de fluorescence à la longueur d'onde d'excitation et/ou de détection.

- 5 10. Le procédé selon au moins l'une des revendications 1 à 9, dans lequel une propriété physique spécifique supplémentaire caractérisant lesdites particules est la durée de vie de fluorescence.
- 10 11. Le procédé selon au moins l'une des revendications 1 à 10, dans lequel une propriété physique spécifique supplémentaire caractérisant lesdites particules est le coefficient de diffusion, ou le temps de corrélation des fluctuations d'intensité de rayonnement, ou toute autre propriété en relation directe avec ledit coefficient de diffusion.
- 15 12. Le procédé selon au moins l'une des revendications 1 à 11, dans lequel les propriétés de fluorescence des particules sont modifiées en conjuguant celles-ci avec une première molécule, en particulier la biotine, qui lie une seconde molécule marquée par fluorescence, en particulier l'avidine ou la streptavidine marquée par fluorescence.
- 20 13. Le procédé selon au moins l'une des revendications 1 à 12, dans lequel les propriétés de fluorescence d'une particule sont modifiées par transfert d'énergie, en sorte que l'énergie absorbée par ladite particule est transférée par contact étroit avec un fluorophore d'un accepteur et émise ensuite.
- 25 14. Le procédé selon au moins l'une des revendications 1 à 13, dans lequel lesdites particules portent chacune un certain nombre de sites de liaison pour particules fluorescentes.
- 25 15. Le procédé selon au moins l'une des revendications 1 à 14, dans lequel le volume de mesure n'est qu'une partie du volume total dudit échantillon et a un volume $\leq 10^{-12}$ l, de préférence $\leq 10^{-14}$ l.
- 30 16. Le procédé selon au moins l'une des revendications 1 à 15, dans lequel lesdites particules se diffusent et/ou sont transportées activement pour entrer dans ledit volume de mesure et en sortir et/ou ledit échantillon est transporté activement et/ou analysé optiquement par balayage.
- 35 17. Le procédé selon au moins l'une des revendications 1 à 16, dans lequel les volumes de mesure ou échantillons sont disposés sur un support bidimensionnel, en particulier sur une membrane ou dans des plaques ayant des puits, ou d'une manière linéaire, de préférence dans un système capillaire.
- 40 18. Le procédé selon au moins l'une des revendications 1 à 17, dans lequel un montage à microscope confocal, comprenant au moins un objectif de microscope, ayant de préférence une ouverture numérique $\geq 0,9$, à la fois pour focaliser un faisceau laser incident et collecter le rayonnement émis par lesdites particules dudit échantillon, un miroir dichroïque, un sténopé dans le plan image dudit objectif de microscope, un moyen de détection, un moyen d'acquisition de données et facultativement des moyens pour analyser par balayage et/ou transporter activement ledit échantillon.
- 45 19. Le procédé selon au moins l'une des revendications 1 à 18, dans lequel ledit volume de mesure est limité par l'utilisation d'éléments de microscopie optique de champ proche, ou de leur combinaison avec une microscopie optique classique.
- 45 20. Le procédé selon au moins l'une des revendications 1 à 19, dans lequel la fluorescence est induite en utilisant une excitation à photons multiples.
- 50 21. Le procédé selon au moins l'une des revendications 1 à 20, dans lequel ladite distribution de la longueur d'intervalle de temps est ajustée en utilisant une information préalable sur ledit échantillon.
- 55 22. Le procédé selon au moins l'une des revendications 1 à 20, dans lequel ladite distribution de la longueur d'intervalle de temps est traitée en appliquant une transformation inverse avec régularisation et/ou contraintes linéaires.
- 55 23. Le procédé selon au moins l'une des revendications 1 à 22, pour son utilisation dans le triage à haut débit, les tests diagnostiques, le contrôle de processus de polymérisation, les processus d'agrégation et de dégradation, le classement de particules, ou le séquençage d'acide nucléique.

The time interval distribution between consecutive photons

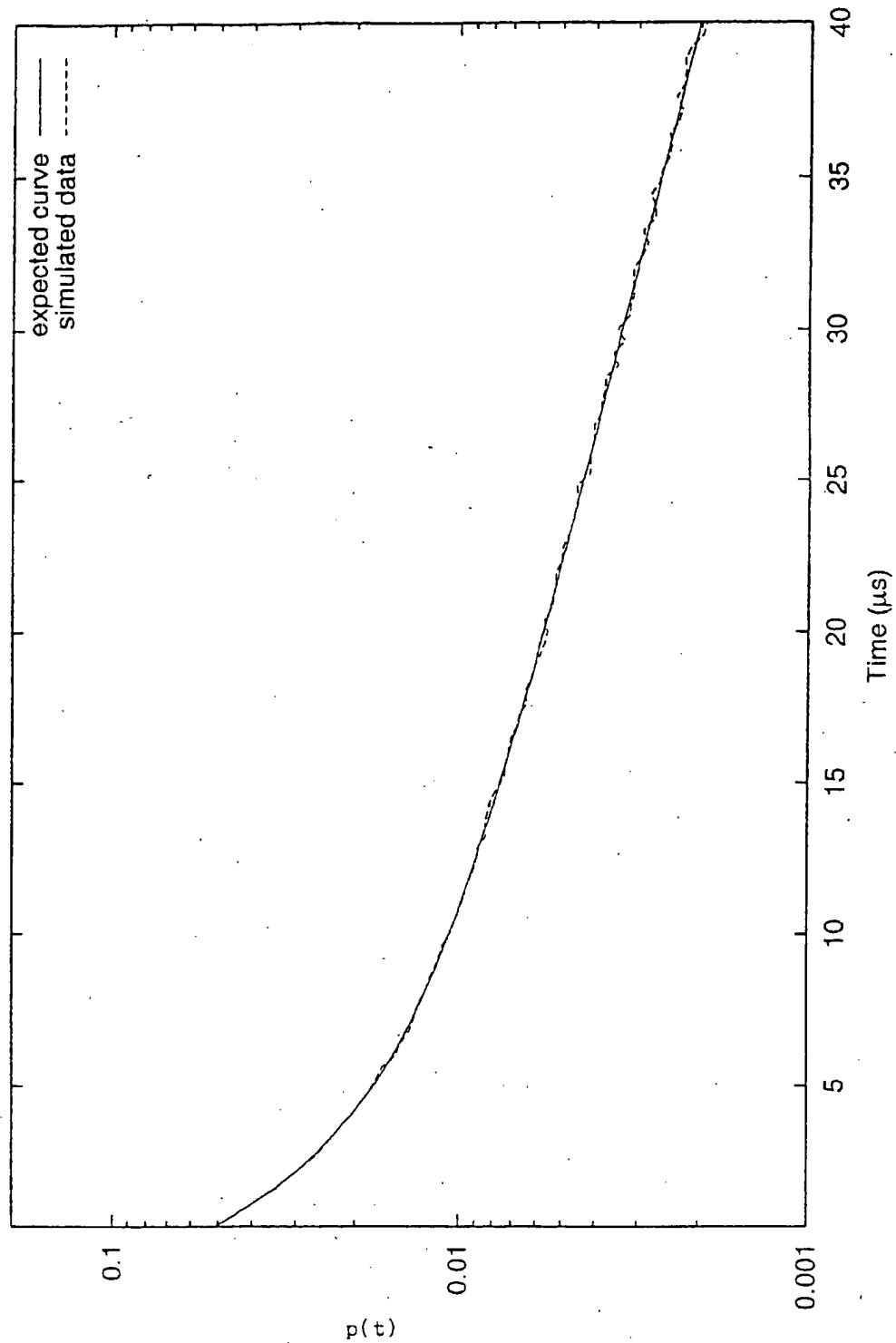


Figure 1

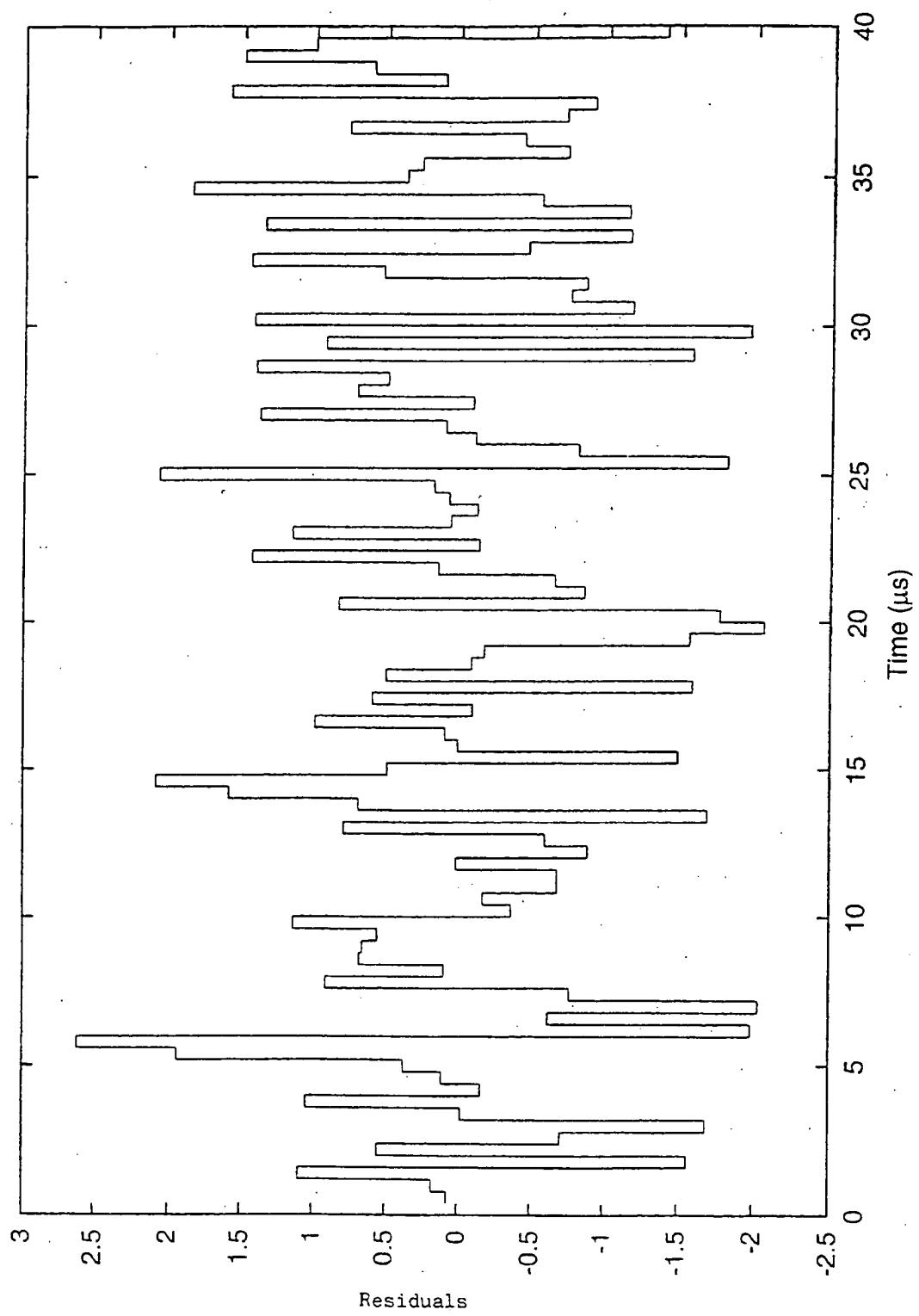


Figure 2